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Phomopsis viticola PCR Detection Kit — Product Insert

Product #34900

Pathogen Information

Phomopsis cane and leaf spot, caused by *Phomopsis viticola*, occurs in most grape-growing regions and is an important disease of grapes worldwide. The fungus attacks all green parts of the vine, including canes, leaves, flowers, rachises, and berries. However, most economic damage is caused by rachis and berry infection. Infected leaves have small, yellowish spots with dark brown centers and may be puckered. With successive cool, wet springs, the inoculum will build up and infections will become more severe over time. Since symptoms appear 21 to 30 days after infection, early detection of the pathogen in the vineyard is therefore crucial for its control.

Principle of the Test

Norgen's *Phomopsis viticola* PCR Detection Kit constituents a ready-to-use system for the isolation and detection of *P. viticola* using end-point PCR. The kit first allows for the isolation of fungal DNA using spin-column chromatography based on Norgen's proprietary resin. Fungal DNA can be isolated from fungi growing on culture plates, or from plant tissue or fruit using this kit. The DNA is isolated free from inhibitors, and can then be used as the template in a PCR reaction for *P. viticola* detection using the provided *P. viticola* Master Mix. The *P. viticola* Mastermix contains reagents and enzymes for the specific amplification of a 300 bp region of the fungal genome. In addition, Norgen's *Phomopsis viticola* PCR Detection Kit contains a second heterologous amplification system to identify possible PCR inhibition and/or inadequate isolation. The amplification and detection of either the *Isolation Control (IsoC)* or the *PCR control (PC)* does not reduce the detection limit of the analytical *P. viticola* PCR. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Lysis Solution	15 mL
Wash Solution	9 mL
Elution Buffer	3 mL
Bead Tubes	24
Mini Filter Spin Columns	24
Collection Tubes	24
Elution tubes (1.7 mL)	24
***P. viticola* 2x PCR Master Mix**	0.35 mL
***P. viticola* Isolation Control (IsoC)*[a]**	0.3 mL
***P. viticola* Positive Control (PosC)*[b]**	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
Product Insert	1

** IsoC = Isolation Control ; PosC= Positive Control*
[a] The isolation and PCR control are a cloned PCR product.
[b] The positive control is P. viticola genomic DNA

Customer-Supplied Reagents and Equipment

- Disposable powder-free gloves
- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 65°C water bath or heating block
- 96 – 100% ethanol
- 70% ethanol
- RNase A (optional)
- Lyticase (optional)

Storage Conditions and Product Stability

All buffers should be kept tightly sealed and stored at room temperature (15-25°C). Buffers can be stored for up to 1 year without showing any reduction in performance.

The *P. viticola* 2x PCR Master Mix, *P. viticola* Positive Control (PosC) and the *P. viticola* Isolation Control (IsoC) should be kept tightly sealed and stored at -20°C for up to 1 year without showing any reduction in performance. Repeated thawing and freezing (> 2 x) should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's *Phomopsis viticola* PCR Detection Kit, including the *Phomopsis viticola* 2x PCR Master Mix, *Phomopsis viticola Isolation Control (IsoC)* and *Phomopsis viticola Positive Control (PosC)* are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's *Phomopsis viticola* PCR Detection Kit is designed for research purposes only. It is not intended for human or diagnostic use.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Safety Information

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Protocol

A. *Phomopsis viticola* Genomic DNA Isolation

Important Notes Prior to Beginning Protocol:

- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all isolation solutions are at room temperature prior to use, and that no precipitates have formed. If necessary, warm the solutions and mix well until the solutions become clear again.
- Prepare a working concentration of the **Wash Solution** by adding 21 mL of 96 - 100 % ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 30 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Lysate can be prepared from either fungi growing on plates, plant tissue or fruit. Please ensure that you follow the proper procedure for lysate preparation in **Step 1a.**
- For the isolation of genomic DNA from fungi growing on plates, **Collection Solution** must be prepared. **Collection Solution** consists of 0.9% (w/v) NaCl prepared with distilled water.
- Preheat a water bath or heating block to 65°C.
- ***P. viticola* Isolation Control (*BC IsoC*)**
    - A *P. viticola* Isolation Control (*IsoC*) is supplied. This allows the user to control the DNA isolation procedure. For this assay, add the *P. viticola* Isolation Control (*IsoC*) to the lysate during the isolation procedure
    - The *P. viticola* Isolation Control (*IsoC*) must not be added to the sample material directly.
    - Do not freeze and thaw the *P. viticola* Isolation Control (*IsoC*) more than 2 times.
    - The *P. viticola* Isolation Control (*IsoC*) must be kept on ice at all times during the isolation procedure.
- The PCR components of the *Phomopsis viticola* PCR Detection Kit should remain at -20°C until DNA is extracted and ready for PCR amplification.

1. Lysate Preparation

a. **Fungi Growing on Plates:** Add approximately 5 mL (volume can be adjusted based on density of fungal growth) of **Collection Solution** (see notes prior to Beginning Protocol) to the plate and gently collect fungal spores and mycelium with an inoculation loop or autoclaved pipette tip, ensuring not to collect any agar debris. Transfer up to 1 mL of washed spores and wet mycelium to a microcentrifuge tube (provided by user).

Fungi from Plant Tissue or Fruit: Wash the tissue or fruit with an appropriate amount of DNAse free water with vortexing. Transfer up to 1 mL of washed spores and wet mycelium to a microcentrifuge tube (provided by user).

b. Centrifuge at 14,000 x g (~14,000 RPM) for 2 minutes to pellet the cells. Pour off the supernatant carefully so as not to disturb or dislodge the cell pellet.

c. Add 500 µL of **Lysis Solution** to the cell pellet. Resuspend the cells by gentle vortexing.

d. Transfer the mixture to a provided **Bead Tube** and secure the tube horizontally on a flat-bed vortex pad with tape, or in any commercially available bead beater equipment (e.g. Scientific Industries' Disruptor Genie™).

e. Vortex for 5 minutes at maximum speed or optimize the condition for any commercially available bead beater equipment.

Note: Foaming during the homogenization is common. This foaming is due to detergents present in the **Lysis Buffer** and will not affect the protocol.

f. Incubate the **Bead Tube** with lysate at 65°C for 10 minutes. Occasionally mix the lysate 2 or 3 times during incubation by inverting the tube.
g. Briefly spin the tube to remove liquid from the cap, and transfer all of the lysate, including cell debris, to a DNase-free microcentrifuge tube (provided by the user) by pipetting. Ensure that the beads are not transferred during the pipetting.
h. Centrifuge the tube for 2 minutes at 14000 × g (~14,000 RPM).
i. Carefully transfer clean supernatant to a new DNase-free microcentrifuge tube (provided by the user) without disturbing the pellet. Note the volume.
j. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix.
k. Proceed to Step 2: Binding to Column

2. Binding DNA to Column

a. Assemble a spin column with one of the provided collection tubes.
b. Add 10 µL of ***P. viticola*** **Isolation Control (*IsoC*)** to the lysate mixture.
c. Apply up to 600 µL of the lysate with ethanol onto the column and centrifuge for 1 minute at 14,000 x g (~14,000 RPM). Discard the flowthrough and reassemble the spin column with the collection tube.

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

d. Depending on your lysate volume, repeat step 2c if necessary.

3. Column Wash

a. Apply 500 µL of Wash Solution to the column and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

b. Discard the flowthrough and reassemble the column with its collection tube.
c. Repeat step 3a to wash column a second time.
d. Discard the flowthrough and reassemble the spin column with its collection tube.
e. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

4. DNA Elution

a. Place the column into a fresh 1.7 mL Elution tube provided with the kit.
b. Add 75 µL of Elution Buffer to the column.
c. Centrifuge for 2 minutes at 200 x g (~2,000 RPM), followed by a 1 minute spin at 14,000 x g (~14,000 RPM). Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for 1 additional minute.

5. Storage of DNA

The purified DNA may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

B. *Phomopsis viticola* PCR Assay Preparation

Notes:

- Before use, suitable amounts of all PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
- The amount of *P. viticola* 2X PCR Master Mix provided is enough for up to 32 PCR reactions (24 sample PCR, 4 positive control PCR and 4 no template control PCR).
- For every PCR run, one reaction containing *P. viticola* Positive Control (*PosC*) and one reaction as no template control must be included for proper interpretation of results.
- The recommended minimum number of DNA samples tested per PCR run is 6.
- Using a lower volume from the sample than recommended may affect the sensitivity of *P. viticola* Limit of Detection.

1. Prepare the PCR for sample detection as shown in Table 1 below. The recommended amount of sample DNA to be used is 5 µL. However, a volume between 1 and 10 µL of sample DNA may be used as template. Adjust the final volume of the PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. PCR Assay Preparation

PCR Components	Volume Per PCR Reaction
***P. viticola* 2X PCR Master Mix**	**10 µL**
Sample DNA	**2 to 5 µL**
Nuclease-Free Water	**Up to 10 µL**
Total Volume	**20 µL**

2. For every PCR run, prepare **one** positive control PCR as shown in Table 2 below:

Table 2. PCR Positive Control Preparation

PCR Components	Volume Per PCR Reaction
***P. viticola* 2X PCR Master Mix**	**10 µL**
P. viticola Positve Control (PosC)	**10 µL**
Total Volume	**20 µL**

3. For every PCR run, prepare **one** no template control PCR as shown in Table 3 below:

Table 3. PCR Negative Control Preparation

PCR Components	Volume Per PCR Reaction
***P. viticola* 2X PCR Master Mix**	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

C. *Phomopsis viticola* PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run PCR.

Table 4. *P. viticola* Assay Program

PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**95°C**	**3 min**
Cycle 2 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**60°C**	**30 sec**
	Step 3	**72°C**	**45 sec**
Cycle 3	**Step 1**	**72°C**	**5 min**
Cycle 4	**Step 1**	**4°C**	**∞**

D. *Phomopsis viticola* PCR Assay Results Interpretation

1. For the analysis of the PCR data, the entire 20 µL PCR Reaction should be loaded on a 1X TAE 1.7% Agarose DNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The PCR products should be resolved on the 1X TAE 1.7% Agarose gel at 150V for 30 minutes.
3. Sample results are provided below:

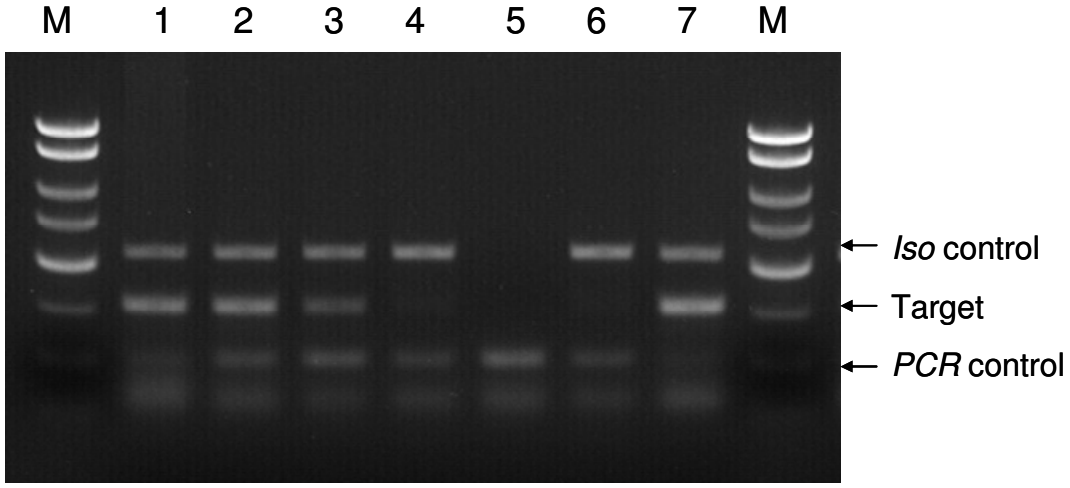

Figure 1: A representative 1X TAE 1.7% agarose gel showing the amplification of *Phomopsis viticola* at different concentrations (Target). The size of the *P. viticola* target amplicon corresponds to 300 bp as represented by the provided DNA Marker (M). The size of the *P. viticola* Isolation Control (*Iso C*) corresponds to 529 bp as represented by the provided DNA Marker (M). The *P. viticola* 2X PCR Master Mix contains a *P. viticola* PCR Control (*PCRC*). The *P. viticola* PCRC controls for PCR inhibition. The size of the *P. viticola* PCRC corresponds to 171bp as represented by the provided DNA Marker (*M*). The amplification from each lane is interpreted as shown below.

Lane 1 to 4: ***P. viticola* detected**: Lanes showed the detection of all three PCR amplicons.
Lane 5: No IsoC and Template– Only ***P. viticola PCRC*** was detected.
Lane 6: *P. viticola* not detected: Detection of ***P. viticola IsoC*** and ***P. viticola PCRC***, suggesting that the DNA isolation was successful but no ***P. viticola*** DNA was present in the sample
Lane 7: Positive: All three PCR amplicons were detected

Table 5. Interpretation of PCR Assay Results

Input Type	*P. viticola Iso C* Band (529 bp)	*P. viticola* Target Band (300 bp)	*P. viticola PCR C* Band (150 bp)	Interpretation
Positive Control	X	X	X	Valid
Negative Control	X		X	Valid
Sample	X	X	X	Positive
Sample	X		X	Negative
Sample	X			Negative
Sample		X	X	Positive
Sample	X	X		Positive
Sample		X		Positive

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. Fungi *Phomopsis viticola* PCR Assay Specificity and Sensitivity

The specificity of Norgen's *Phomopsis viticola* PCR Detection Kit is first and foremost ensured by the selection of the *P. viticola*-specific primers, as well as the selection of stringent reaction conditions. The primers were checked for possible homologies to all in GenBank published sequences by sequence comparison analysis. The specific detectability of all relevant strains has thus been ensured by a database alignment and by PCR amplification with the following fungi commonly found in plant samples:

- *Aspergillus niger*
- *Cladosporium sp.*
- *Phomopsis viticola*
- *Mucor racemosus*
- *Alterneria tenuissima*
- *Rhizopus oryzae*
- *Penicillum sp.*
- *Fusarium oxysporum*

F. Linear Range

- The linear range (analytical measurement) of Norgen's *Phomopsis viticola* PCR Detection Kit was determined by analysing a dilution series of a *P. viticola* quantification standards ranging from 1 x 10^7 cfu/µl to 1 x 10^3 cfu/µl.
- Each dilution has been tested in replicates (n = 4) using Norgen's *Phomopsis viticola* PCR Detection Kit on a 1X TAE 1.7% agarose gel.
- The linear range of Norgen's *Phomopsis viticola* PCR Detection Kit has been determined to cover concentrations of genomic DNA from 10 pg to 100 ng
- Under the conditions of the Norgen's *Phomopsis viticola* DNA Isolation procedure, Norgen's *Phomopsis viticola* PCR Detection Kit covers a linear range from 500 copies to 5 x 10^6 copies.

Frequently Asked Questions

1. How many samples should be included per PCR run?

- Norgen's *Phomopsis viticola* PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control (Nuclease Free Water) and a Positive Control must be included. It is preferable to pool and test 6 samples at a time. If not, the provided Positive Control is enough to run 3 samples at a time.

2. How can I interpret my results if neither the *P. viticola* PCR control nor the *P. viticola* Isolation Control (IsoC) amplifies?

- If neither the *P. viticola* PCR control nor the *P. viticola* Isolation Control (IsoC) amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the Problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the *P. viticola* PCR control showed amplification but neither the *P. viticola* target nor the *P. viticola* Isolation Control (IsoC) amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the *P. viticola* Isolation Control (IsoC) was amplified in a sample?

- The sample tested can be considered as *P. viticola* negative.

5. How should it be interpreted if the *P. viticola* PCR control and the *P. viticola target* showed amplification in a sample?

- The sample tested can be considered positive. It could happen when Isolation Control failed to be amplified due to the incorrect spiking volume of Isolation Control or low Isolation Control recovery in the elution with high *P.viticola* input.

6. How should it be interpreted if only the *P. viticola* target and the *P. viticola* PCR control were amplified in a sample?

- The sample tested can be considered as *P. viticola* positive.

7. How should it be interpreted if only the *P. viticola* target was amplified in a sample?

- The sample tested should be considered as *P. viticola* positive. At high *P. viticola* cell input, the *P. viticola* amplicon will be predominant and thus the *P. viticola* PCR control as well as the *P. viticola* Isolation control may not amplify as they compete for PCR resources.

8. How should it be interpreted if only the *P. viticola* PCR control and the *P. viticola* Isolation Control (IsoC) showed amplification in a sample?

- The sample tested can be considered negative

9. What If I forgot to do a dry spin after my second wash?

- Your first DNA elution will be contaminated with the Wash Solution. This may dilute the DNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

10. What If I forgot to add the *P. viticola* Isolation Control (IsoC) during the isolation?

- It is recommended that the isolation is repeated.

Related Products	Product #
Bacterial Genomic DNA Isolation Kit	17900
Fungi/yeast Genomic DNA isolation kit	27300
Plant/Fungi DNA Isolation Kit	26200

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Urine DNA Isolation Mini Kit (Slurry Format) or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362 or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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